

THE ISOLATION OF OLIGOGALACTURONIC ACIDS BY DEAE-SEPHADEX A-25 COLUMN CHROMATOGRAPHY*

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The isolation of oligogalacturonides from the acid and enzymic digests of pectic substances is needed in the structural analysis of pectic substances. Furthermore, the oligouronides are required as substrates for the studies of polygalacturonases or pectic acid transeliminases. In the previous study (1), galacturonides ranging in degree of polymerization from 1 to 6 could successfully be separated from one another by DEAE-cellulose column chromatography with an acetate buffer and sodium bicarbonate solutions, or with a linear gradient of increasing concentration of sodium bicarbonate. In the present work we show that, by using DEAE-Sephadex A-25 in place of DEAE-cellulose and ammonium bicarbonate solution as eluting agent, oligogalacturonides of chain length from 2 to 9 units are separated from one another. By this method good separations are obtained also between digalacturonic acid and 4,5-unsaturated digalacturonic acid, which were unable to be resolved by the DEAE-cellulose chromatography with sodium bicarbonate.

MATERIALS AND METHODS

1. *Mixture of Oligogalacturonides*

Endopolygalacturonase. *Saccharomyces fragilis* was grown stationarily in 100 ml of a medium in 300-ml Kolle flasks at 27°C (2). The medium contained 5 % glucose, 0.2 % $\text{NH}_4\text{H}_2\text{PO}_4$, 0.2 % KH_2PO_4 , 0.1 % MgSO_4 and 1 % dried yeast. After 3 days' incubation, the culture fluid was centrifuged to remove yeast cells and the supernatant was dialyzed against 0.01 M acetate buffer, pH 5.0, for 4 days. The dialyzed solution was added to a column (4×5 cm) of Amberlite IRC-50 (200~400 mesh) which had been equilibrated with 0.02 M acetate buffer, pH 5.0. The column was washed with the same buffer until the effluent gave no phenol-sulfuric acid test (3) for sugars and then eluted with 1 M sodium acetate. The eluate was collected in 100 ml fractions and assayed for the enzyme activity toward pectic acid. The fractions showing the enzyme activity were combined, concentrated by pervaporation and dialyzed against 0.02 M acetate buffer, pH 5.0.

Mixture of Oligogalacturonides. The mixture containing 350 ml of 2 % pectic acid (pH 5.0) and 5 ml (420 units) (4) of the polygalacturon-

*This is the English edition of the article published in Nippon Nogeikagaku Kaishi (J. Agr. Chem. Soc. Japan) 46: 417-420 (1972).

ase preparation (pH 5.0) was incubated for 2 hours at 35°C. The reaction was stopped by the addition of 5 % HCl, the pH of the mixture being lowered to 2.0~2.3. The enzyme was removed by passing a column of Amberlite IRC-50 (2×10 cm) previously equilibrated with 0.02 M acetate buffer, pH 3.3, and one-fifteenth volume of 15 % CuSO₄ was added to the effluent. The mixture was brought to pH 4.5 by the addition of 1 N NaOH and left overnight at 4 to 5°C. The precipitate formed was collected by centrifugation, washed with cold water and suspended in water. The suspension was freed from cupric ions by the treatment with a cation exchange resin, neutralized with sodium hydroxide solution and concentrated under reduced pressure (mixture of oligogalacturonides). The pectic acid described above was made from a commercial preparation of citrus pectinic acid (5).

2. *Chromatography of Oligogalacturonides*

To DEAE-Sephadex A-25 column previously equilibrated with 0.3 M ammonium bicarbonate was added the mixture of oligogalacturonides. The column was eluted with a linear concentration gradient of 0.3 M to 0.6 M ammonium bicarbonate. Sugar was determined by the phenol-sulfuric acid method (3).

For the separation of digalacturonic acid and unsaturated digalacturonic acid too a DEAE-Sephadex A-25 column was used. The column was equilibrated with a 0.1 M solution of ammonium bicarbonate, loaded with the sample and eluted with a linear concentration gradient of the same salt. Digalacturonic acid and unsaturated digalacturonic acid used in this experiment were prepared in the same manner as described in the previous paper (6).

Chromatography of the same samples, namely, the mixture of oligogalacturonides and of digalacturonic acid and 4,5-unsaturated digalacturonic acid, on DEAE-Sephadex A-25 was done also with linear concentration gradient of sodium bicarbonate.

3. *Analytical Methods*

Degree of polymerization of oligogalacturonides. Total galacturonic acid was determined by the carbazole method (7). The reducing galacturonic acid of chain end of oligogalacturonides was estimated by a modification of the Willstätter-Schudel method (8). The degree of polymerization was obtained from the ratio of total galacturonic acid to reducing galacturonic acid of chain end of oligogalacturonides.

Paper chromatography. For the identification of the uronides ascending chromatography was done on strips of Toyo Roshi No. 52 paper, using *n*-butanol-acetic acid-water (5:2:3) as solvent. The spray reagents were aniline-hydrochloric acid for sugars, bromophenol blue for acids and quinine sulfate (9) for 4,5-unsaturated digalacturonic acid.

RESULTS AND DISCUSSION

1. *Separation of Oligogalacturonides*

When 1 and 3 ml of the mixture of oligogalacturonides were chromatographed on the DEAE-Sephadex A-25 with the gradient of ammonium bicarbonate, considerably clear separations into nine components were obtained as shown in Fig. 1. Loading 10 ml of the sample gave a slightly poor resolution as compared with loading 1 or 3 ml (Fig. 1 C).

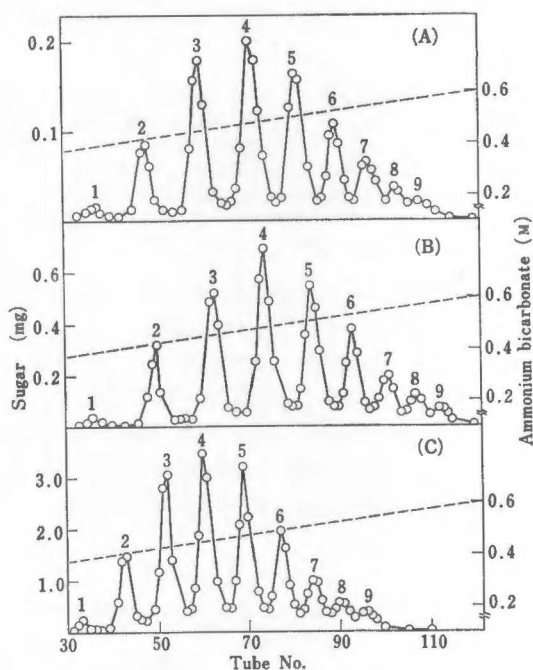


Fig. 1. Column chromatography of oligogalacturonides. The mixture (ca. 1.5 %; A, 1 ml; B, 3 ml; C, 10 ml) of oligogalacturonides was loaded on a column (1.8×45 cm) of DEAE-Sephadex A-25 equilibrated with 0.3 M ammonium bicarbonate. The column was eluted with the following linear gradient of ammonium bicarbonate: in the mixer, 300 ml of 0.3 M ammonium bicarbonate; in the reservoir, 300 ml of 0.6 M ammonium bicarbonate. Dash line, concentration of ammonium bicarbonate. Tube volume, 5 ml.

Fig. 2 shows the chromatograms obtained with the mixture of oligogalacturonides when the column was equilibrated with the solution of sodium bicarbonate and eluted with the concentration gradient of the same salt. Though nine peaks appeared the resolution was considerably poor.

The fractions under each peak (Fig. 1 C) were pooled and purified by rechromatography. They were freed from ammonium ions by treating with a cation exchange resin, concentrated under reduced pressure and

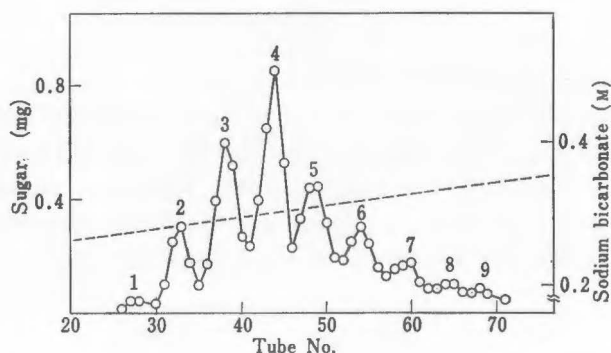


Fig. 2. Column chromatography of oligogalacturonides. The mixture (ca. 1.5 %, 3 ml) of oligogalacturonides was loaded on a column (1.8×45 cm) of DEAE-Sephadex A-25 equilibrated with 0.25 M sodium bicarbonate. A linear gradient was applied to the column by allowing 300 ml of 0.25 M sodium bicarbonate to mix, with constant stirring, with 300 ml of 0.4 M sodium bicarbonate. Dash line, concentration of sodium bicarbonate. Tube volume, 5 ml.

rechromatographed on DEAE-Sephadex A-25 columns (1.8×40~45 cm) equilibrated with 0.3 M ammonium bicarbonate. For elution the following linear concentration gradients of ammonium bicarbonate were used: 0.40 M~0.55 M for peak Nos. 2~4, 0.45 M~0.60 M for peak Nos. 5 and 6 and 0.50 M~0.65 M for peak Nos. 7 and 8; the volume of ammonium bicarbonate solutions in the mixers and reservoirs was 300 ml. The degrees of polymerization of oligogalacturonides in the peaks eluted are shown

TABLE 1
Chain length of the oligogalacturonic acids

Peak number	Ratio of uronic acid to aldehyde group content
2	3.08
3	3.95
4	5.06
5	6.10
6	6.92
7	7.88
8	8.86

Uronic acid and aldehyde group contents were determined by the carbazole method (7) and by a modification of the Willstätter-Schudel method (8), respectively.

in Table 1. Peak Nos. 2~8 correspond to the trimer~nonamer. The oligouronide in peak No. 1 was identified as digalacturonic acid by paper chromatography.

Recently Nagel and Wilson (10) have separated the monomer~octamer by Dowex-1 X 8 column chromatography with sodium formate solution as eluent. The sample added to the column was a digest equivalent to

30 g of pectic acid. Since the column had a volume of about 750 ml representing about 1000 mequiv. and the digest contained about 170 mequiv. of carboxyls, the column load was 17 % of capacity. DEAE-Sephadex A-25 column (1.8×40 cm) had a volume of 102 ml representing about 40 mequiv. and 1 ml of the mixture of oligogalacturonides contained about 0.08 mequiv. of carboxyls. The column load was calculated to be only 0.2 % of capacity when 1 ml of the sample was added. It was as low as 2 % even 10 ml of the sample was used. Considering the results in Fig. 1, the column load should be below 2 % of capacity, otherwise poor resolutions will result.

2. Separation of Digalacturonic Acid and 4,5-Unsaturated Digalacturonic Acid

Our attempt to separate digalacturonic acid and 4,5-unsaturated digalacturonic acid on DEAE-cellulose columns has never been successful because of the similarity in chromatographic behavior between them. When DEAE-Sephadex A-25 columns were used, good separations were obtained between the two oligouronides as shown in Fig. 3. Digalac-

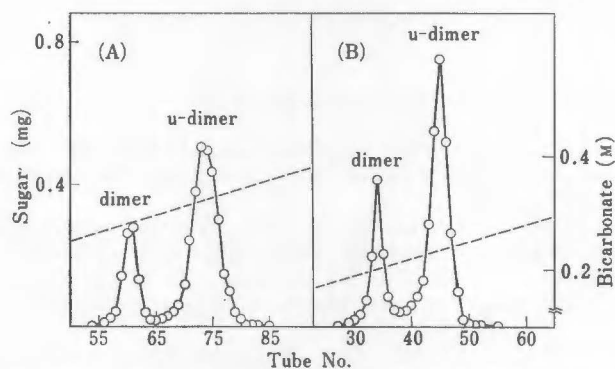


Fig. 3. Column chromatography of digalacturonic acid and 4,5-unsaturated digalacturonic acid. The mixture composed of 1 part of *ca.* 2% digalacturonic acid and 3 parts of *ca.* 2% 4,5-unsaturated digalacturonic acid (1 ml) was loaded on a column (2×20 cm) of DEAE-Sephadex A-25 equilibrated with 0.1 M ammonium bicarbonate (A) or 0.1 M sodium bicarbonate (B). A linear gradient was applied to the column by allowing 250 ml of 0.1 M ammonium bicarbonate (A) or 0.1 M sodium bicarbonate (B) to mix, with constant stirring, with 250 ml of 0.4 M ammonium bicarbonate (A) or 0.4 M sodium bicarbonate (B). Dimer, digalacturonic acid; u-dimer, 4, 5-unsaturated digalacturonic acid. Dash line, concentration of ammonium bicarbonate (A) or sodium bicarbonate (B). Tube volume, 5 ml.

turonic acid had a higher mobility than 4,5-unsaturated digalacturonic acid. They can be distinguished from each other also on the basis of the following points.

- 1) On paper chromatography digalacturonic acid gives a spot with R_f value lower than 4,5-unsaturated digalacturonic acid.
- 2) On spraying with the aniline-hydrochloric acid reagent, the spot of digalacturonic acid gives a red color; that of 4,5-unsaturated digalacturonic acid gives an orange color with the same reagent.
- 3) The spot of digalacturonic acid does not react toward the quinine sulfate reagent; that of 4,5-unsaturated digalacturonic acid reacts positively.
- 4) Digalacturonic acid is negative to the thiobarbituric acid test (11); 4,5-unsaturated digalacturonic acid is positive.

SUMMARY

Oligogalacturonides ranging in degree of polymerization from 2 to 9 were separated from one another by the DEAE-Sephadex A-25 column chromatography with a gradient of increasing concentration of ammonium bicarbonate. Digalacturonic acid and 4,5-unsaturated digalacturonic acid, which had been unable to be resolved by the DEAE-cellulose chromatography with sodium bicarbonate, were separated clearly by the chromatography on DEAE-Sephadex A-25 columns.

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